Final Report on a Research Grant from The Rheumatoid Disease Foundation.

Title of Grant: "Isolation and cultivation of soil amoebae from fluids and tissues of patients with rheumatoid disease."

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INTRODUCTION

Roger Wyburn-Mason hypothesized that a variety of rheumatoid diseases (RD), including rheumatoid arthritis (RA), as well as tumors of human and animal origin, were caused by a soil amoeba (25, 26, 27). He named the amoeba *Amoeba chromatosa*. The main evidence for his hypothesis was that he claimed he had isolated the amoeba from all of his RD patients.

Research performed, during the tenure of this grant, was restricted to an attempt to confirm Wyburn-Mason's claim that living amoebae were found in, and could be isolated from, all cases of RD. The material studied was limited to synovial fluid (SF) aspirated by 15 physicians in 11 states from joints of 35 patients suffering from various types of RD. Samples were studied in the following ways: examination of the fresh samples with phase contrast and polarization microscopy; study of fixed and Giemsa stained slides by brightfield microscopy; culture of aliquots of each sample on 5 media, each at 3 temperatures; and immunological studies of agglutination and lysis of *Acanthamoeba*.

Much of Wyburn-Mason's published work was with solid tissue (25, 26). Tissue was minced, placed in a thermal migration apparatus, and the cells from the warm side of the filter allowed to settle out of the amoeba saline. He reported that even low speed centrifugation killed the amoebae. Cells recovered by settling, at 1 g, were used successfully to culture amoebae on Noble agar, carpeted with living *E. coli*. Identification of amoebae, concentrated by thermal migration, was by microscopic examination of wet preparations. The type of optics used was not discussed. Judging from the quality of the photomicrographs (26, pp 122-123) brightfield was used. He described amoebae as being similar in appearance to that of Acanthamoeba and Naegleria but larger. The trophozoites were 25 to 30 microns in diameter. He believed the amoebae were contractile in that they were able to pass through the fine pores of the filter of the thermal migration apparatus (.5 to 1.0 microns in diameter, average pore size .8 microns). The amoebae were usually light red to dark brown when isolated. If initially clear, they became light red then brown with time (25, 26). Amoebae had 1 to 12 contractile vacuoles. In stained tissue sections, amoebae were difficult or impossible to distinguish from macrophages or lymphocytes. Cysts were identified as 9 to 30 microns in diameter, spherical, dark brown or black, and with fenestrated walls. Encystment did not occur in tissues but only in vitro in amoeba saline. Cysts formed clumps in amoeba saline. When amoebae were placed in distilled water, they became biflagellated.

In a footnote (27, p 5), Wyburn-Mason refers to a method of isolating amoeba cells from SF of inflamed rheumatoid joints by allowing the cells to settle out by gravity alone. The method was developed and used by P. K. Pybus of South Africa. Wyburn-Mason observed that the sediment contained amoeba cysts of the same structure as those shown in photomicrographs in his monograph. He noted these cysts were colored also, *Pybus and Davies later concluded they were viewing macrophages not amoebae*. Wyburn-Mason's results on identification and culture of *Amoeba chromatosa* have not been confirmed in publication. In his monographs (26, 27) he stated that two workers confirmed his findings and notified him in personal communications. Their results have not been published and thus Wyburn-Mason's claims remain to be validated. The present research does not support his claims for finding amoebae in SF of RD patients.

MATERIALS AND METHODS,

Media used. The following dehydrated media from Difco were prepared as indicated by the manufacturer: Sabouraud's Dextrose Agar (SDA); Fluid Thioglycollate Medium (FTM); Nutrient Broth (NB); and Nutrient Broth Agar (NBA). NB and NBA had NaCl
added to a final concentration of .5 % (w/v). All were sterilized by autoclaving 15 min at 15 lbs.

Two tissue culture media from Gibco were used. They were Eagle's MEM and RPMI-1640. They were dehydrated. They were reconstituted with distilled water, filter sterilized, and enriched to 10 % with fetal calf serum from Gibco.

Two liquid media for the axenic cultivation of amoebae were prepared in the laboratory. The first medium (17), suitable for a wide variety of non-Naegleria soil amoebae, was Neff's growth medium (NGM). The final concentration of each ingredient was: proteose peptone (Difco), .75 %; yeast extract, .75 %; glucose, 1.5 %; MgSO₄, 1 mM; CaCl₂, .05 mM; KH₂PO₄, 2 mM; ferric citrate, .1 mM; B₂ hydrochloride, 1 mg/L; biotin, .2 mg/L; B¹₂, 1 microgram/L; and phenol red, .0008 %. After all ingredients were dissolved in water, the pH was adjusted to 7.4 and sterilized by autoclaving.

The second liquid medium was Fulton's B medium (FBM) for Naegleria (11). The final concentration of each constituent was: proteose peptone (Difco), 1 %; yeast extract, (Difco), .5 %; glucose, .03 M; liver concentrate NF (Pfaltz and Bauer, Inc.), 1 %; KH₂PO₄, 5 mM; Na₂HPO₄, 5 mM; and fetal calf serum (Gibco), 10 %. The first six ingredients were prepared as concentrated solutions, sterilized by autoclaving, and the proper amount added aseptically to the fetal calf serum. The liver concentrate was prepared as a 5 % aqueous solution and autoclaved in screwcap centrifuge tubes. The large precipitate was removed by centrifugation, 2,400 X g for 10 min, and the clear supernatant decanted and reautoclaved. For making the final FBM, the clear liver concentrate was treated as if it contained 5 % solids.

Monoxenic plate growth of all types of amoebae was on a thin carpet of bacteria growing on Schuster's (21) amoeba agar (SAA). The final concentration of each ingredient in SAA was: proteose peptone (Difco), .05 %; yeast extract (Difco), .05 %; glucose, .1 %; agar, 1.5 %; MgSO₄, 4 mM; CaCl₂, .4 mM; Fe(NH₄)₂(SO₄), .05 mM; Na₃PO₄, 1 mM; and KH₂PO₄, 4 mM. The medium was autoclaved 15 min at 15 lbs. The SAA was cooled to 57°C before adding to tubes or plates. In order to make a medium that was approximately isotonic for human cells, as well as for amoebae, Schuster's formulation was made .1 M in NaCl for most of the studies.

Amoeba saline. Three salines were used in the present study. The first was a saline designed by Page (18). It was a modification of an early saline concocted by the author (16) but since abandoned. Page's saline (PS) has the following composition given in grams per liter: NaCl, .12; MgSO₄·7H₂O, .004; CaCl₂·2H₂O, .004; Na₂HPO₄, .142; and KH₂PO₄, .136. The resulting pH was 6.8 to 7.0. It was autoclaved 15 min at 15 lbs. PS was used only in attempts to repeat Wyburn-Mason's thermal migration procedure.

A basal salt solution (BSS), used in most of the centrifugal washings, evolved slowly in this laboratory. Although previously unpublished, it has been used with amoebae, various other protozoans, bacteria, tissue culture cells, tumor cells, and blood cells, without adverse effects. Soil amoebae will encyst in it, and blood cells will survive in it for several days at room temperature. In composition BSS is: NaCl, .14 M; TRIS (tris-hydroxy-methyl-amino methane), .01 M; MgSO₄, .001 M; CaCl₂, .00005 M. After adjusting the pH to 7.4 with 6 N HCl, the solution is autoclaved 15 min at 15 lbs.

A dilution saline (DS) was developed for the immunological studies. In composition it is: NaCl, .14 M; citric acid, .0025 M, and Na₂HPO₄, .005 M. The pH was adjusted to 5.0 before autoclaving.
Culture containers. Culture vessels were of two types mainly 16 X 125 mm screwcap test tubes (Pyrex) and 60 X 15 mm plastic tissue culture dishes (Falcon). Tubes with solid slanted agar (SDA, NBA, and SAA) contained 5 ml of medium. Tubes with liquid media (FBM, NGM, and NB) contained 2 ml. They were incubated in a horizontal position, with caps unscrewed a fraction of a turn, in order to improve gas exchange. FTM is a semisolid agar medium and contains an oxygen scavenger, thioglycollate. Tubes contained 5 ml and were incubated in an upright position, with minimum agitation, and with screwed-down caps, to reduce or eliminate gas exchange. Plates contained 5 ml of medium.

Cell types. Five axenic strains of soil amoebae were used as control cells in growth, staining, thermal migration, and immunological studies. They were Naegleria gruberi (strains NB-1, and EgB); Acanthamoeba castellanii (strains I1-12, and I2-3B2); and Hartmanella culbertsonii (strain A-5). Hartmanella culbertsonii is also known as Acanthamoeba culbertsonii. All are free-living amoebae. H. culbertsonii is pathogenic for mice. Naegleria were maintained in FBM or on bacterized slants of SAA. Acanthamoeba and Hartmanella strains were maintained in NGM.

Two bacterial species were used as the food organisms on the SAA plates and tubes. They were Escherichia coli (strain C-600) and Klebsiella pneumoniae (previously called Aerobacter aerogenes). They were maintained on NBA slants or in NB.

Blood from finger puncture of the principal investigator was used to make fresh blood smears for stain controls.

Sample handling. Samples were received from local sources .5 to 3 hours after aspiration. Samples from mail-in sources arrived 3 to 6 days after taking. Samples received late in the day were placed in the refrigerator over night at 3 to 4° C. Inoculation of media, direct observation of fresh material, and smearing of slides for later staining required at least 6 hours per sample.

Direct microscopic examination of synovial fluid. Wet preparations of samples were examined immediately after arrival. Drops of fluid were placed in a Levy Ultra Plane blood cell counting chamber. The chamber was .1 mm deep and without an undersurface concavity. Samples were observed with a phase contrast microscope, equipped with a long working distance objective, at 400 X magnification. One of the two chambers contained undiluted SF, and the second SF diluted 1 to 10 with BSS or PS. All observations were made in a 29° C room. At this temperature viable white cells and control amoebae extended pseudopods. Some SF white blood cells would attach and spread out. Cell counts were made on the dilute samples. Total white and red blood cell counts were made and the proportion of dead white blood cells determined. Amoebae trophozoites and cysts were searched for. Additionally, fibres or fibre bundles, abnormal red cells, crystals, and unusual intracellular inclusions were noted (3, 13, 19, and 20).

Living and spread soil amoebae were easily recognized with phase contrast optics at 200 to 400 X due to their unique nuclear and cytoplasmic structures. Their nuclei have a large central nucleolus. This is in sharp contrast to mammalian nuclei which are usually larger and appear uniform. When mammalian nucleoli can be seen, they are usually smaller, multiple, and not centrally located. An amoeba cytoplasm is apt to be more vacuolated, particularly if it has been feeding by pinocytosis. One cannot distinguish between amoebae and white blood cells on the basis of pseudopod form in most cases. Both extend lobose and filar pseudopods. However, macrophages do produce ruffled pseudopods which are seldom seen in amoebae. Neither amoebae or white blood cells can be identified if they remain rounded. For rounded cells the only solutions are to make thin slide coverslip preparations or to smear and stain.
Cysts were identified with phase contrast by the presence of a thickened cyst wall. Additionally cyst walls can be identified with a polarizing microscope because they are highly birefringent. This is due to the cellulose component of the wall. In the present study, any structure that might have been a cyst or piece of cyst wall was examined, at the same magnification, in a polarization microscope for the presence and sign of birefringence. Also, any structure that appeared to be crystalline or fibrous was examined under crossed nicols.

Most dead cells were distinguished easily from living cells in phase contrast due to a marked difference in refractive index. Changed refractive index is due to loss of soluble materials through ruptured portions of the plasma membrane and is identified by the change in phase contrast - particularly in the cytoplasm. Certainly the phase contrast in living and dead cells is different and easily identified. There is close correspondence between cells identified as dead by phase contrast and those that are identified as dead because they cannot exclude a large dye molecule such as trypan blue.

Inoculation of growth media. Samples with abundant cells were inoculated directly into media, usually 1 drop. For samples with few cells, and suitable sample volumes, 4 to 5 ml aliquots were centrifuged at 600 X g for 10 min. The concentrated cells and debris were used as inoculum. Sometimes the concentrated material was used for direct microscopic examination.

Bacterized plate cultures required freshly prepared plates. After receipt of a sample. SAA was melted, cooled, and 5 ml aliquots pipetted into 8, 60 X 15 mm plates. After cooling and solidifying, half of the plates were covered (7 to 8 drops) with an overnight culture of E. coli. The other four were covered with K. pneumonia. Both bacteria were grown in NB. Excess culture fluid was withdrawn with sterile Pasteur pipets. Plates were allowed to dry for 20 to 30 min then inoculated in the center with one drop of SF or concentrated cells.

Cultures, growth conditions and surveillance for growth. Three tubes each of the 4 media, FBM, NGM, SDA, and FTM, were inoculated with SF for each sample. For a given medium, 1 tube was incubated at 19, a second at 29, and the third at 37°C. Six plates, 3 spread with E. coli and 3 with K. pneumonia, were inoculated with the sample. For each bacterial type, 1 plate was incubated at 19, a second at 29, and the third at 37°C. The 2 remaining plates, 1 with E. coli and the other with K. pneumonia, were inoculated with an in-house strain of soil amoeba and incubated at 29°C. These controls were to make certain that the 2 bacterial cultures continued to be suitable food organisms. Plates were incubated in an inverted position in a water-saturated atmosphere.

Tube cultures were checked by eye, at 2 or 3 day intervals, for macroscopic signs of growth. This was continued for at least a month. Growth on plates was checked at 3 to 4 day intervals using an inverted microscope with phase contrast optics at 100 X magnification. Laboratory strains of amoebae and cysts, as well as blood cells, could be identified with the inverted microscope at this magnification.

Approximately a month after inoculation, all cultures were examined with phase contrast at 400 X magnification, in counting chambers, as described above for direct microscopic examination. Materials on agar plates were examined by adding a few drops of BSS to the surface with a Pasteur pipet. The drops were mixed with the surface material by sucking in and forcing out the fluid on the surface. A drop of the mixture was added to the counting chamber. After the final examination, all tubes and plates were autoclaved before cleaning or discarding. Pipets used in transferring specimens of SF were soaked in 2.5 % Lysol and/or autoclaved before cleaning.
Three of the samples were inoculated into Eagle's MEM and RPMI-1640. Each plate contained 5 ml of one of these liquid media. Plates were incubated at 37° C in an atmosphere of 5 % CO₂ and 95 % air. Cultures were examined periodically over a month with the inverted microscope.

Fixed and stained preparations. Giemsa staining was selected as the routine stain. A drop of SF was spread on each of 4 to 8 slides with the tip of a Pasteur pipet. Slides were allowed to dry in a warm current of air at 29° C so that they dried in 30 to 60 seconds. They were fixed in 100 % methanol for 3 or more minutes and were then air dried until they were stained.

Giemsa stock solution (6, 12, 13) was prepared by adding .5 g of dry powdered stain (Allied Chemical or Fisher) to 33 ml of CP glycerin and heated at 55 to 60° C for 2 hours. On addition of 33 ml of absolute methanol and mixing, the stain was ready for use. The procedure for staining was as follows: To 2.5 ml of the stock Giemsa solution was added 1.5 ml of methanol and 46 ml of 0.1 M phosphate or phosphate-citrate buffer, pH 6.5 to 7.2, freshly diluted from .1 M stock buffer. (In this range of pH it was possible, with most brands of Giemsa dye, to find a pH where it was possible to identify easily the various white blood cell types and distinguish them from the amoebae.) The diluted Giemsa stain was poured over slides in a Coplin jar and stained for 5 to 15 min with frequent slide agitation. Staining time was constant for a given batch of stain once the optimal time for staining was determined on test slides of blood and amoebae. Slides were dipped in 2 or 3 successive Coplin jars of fresh distilled water and left to dry in a vertical position. Slides were examined at 1,000 X with an oil immersion lens and brightfield optics.

All samples received were viscous due to synovial mucin (hyaluronic acid). Synovial mucin appeared as amorphous material on the stained slides. It prevented many cells from flattening and thus made identification of cells, on the basis of differential staining of the intracellular morphology, difficult. Good flattening of cells was achieved by diluting an aliquot of SF 10 times with BSS, mixing and centrifuging 10 min at 600 X g. Smear slides prepared with these washed and concentrated cells were labeled "concentrated." As many as 8 to 16 slides were prepared from some samples, half were from undiluted and half from concentrated SF. To assure reliable staining of samples, fresh smears of blood and amoebae were stained with the sample slides in the same Coplin jar.

Two additional stains were tested. They were Wright's stain (Harleco and U. S. Biochemical Corp.) and hematoxylin (Sargent and U. S. Biochemical Corp). The methods used for Wright's stain (12, 13, or directions provided by the manufacturer) proved to be satisfactory for blood cells but were unreliable for amoebae and cysts. The hematoxylin methods used (6, 12, 13) did not yield slides that permitted reliable identification of either blood cell types or the nuclear and cytoplasmic structures of amoebae.

Immunological studies. Antibodies against cell surface antigens of soil amoebae were identified by reacting serial dilutions of SF with a standard number of living soil amoebae. Presence of antibody was detected by agglutination or by lysis of the living cells. Methods used were developed in this laboratory. The methods selected, after experimentation, are given below.

The test organism used was Acanthamoeba castellanli, strain 12-3B2. It was the in-house soil amoeba that showed no self agglutination on standing for extended periods in the DS. Cells were grown in aerated cultures in NGM, while still in the exponential phase of growth, cells were counted, aliquots taken, washed twice centrifugally in DS at 600 X g, suspended at 10⁶ cells per ml, and stored briefly on ice.
Agglutination tests were performed in transparent spot plates (Pyrex, 7720) with 9 concave wells. A single drop of DS was placed in wells 2 through 9. A single drop of SF was placed in each of the first and second wells. The drops in the second well were mixed, and a drop of the mixture transferred to the third well. Mixing and transferring to following wells was continued to the eighth well. After mixing the contents of the eighth well, a drop was discarded. There was then a series of two fold dilutions of SF in the first eight wells ranging from no dilution in the first well to 1 to 128 dilution in the eighth well. The ninth well with one drop of DS was to serve as a self-agglutination control. Cells in the iced DS were mixed, 1 drop added to each of the nine wells, and the contents mixed by gently rotating the plate in a horizontal plane. Dilution of SF now spanned the range from 1 to 2 in the first well to 1 to 256 in the eighth well. Wells were observed for agglutination immediately, at 1.5 hours, and at 3 hours. Observations were made with a stereomicroscope at 40 X magnification using transmitted light. Agglutination was scored by the size and tightness of clumps. Scoring ranged from 0 (no clumps, all single cells) to 4 (majority of cells in large tight clumps). Between observations, plates were covered tightly with polyethylene sandwich bags to prevent evaporation. Tests were performed at 25° C.

Lysis was assayed after the last observation of agglutination. Drops were removed from wells beginning at the highest SF concentration (1 to 2 dilution). Wells of decreasing concentration were sampled until a well was found with no lysis. Observations were made in counting chambers at 400 X with phase contrast optics. Lysed cells were generally intact but had frayed borders. The phase contrast of the cytoplasm was changed markedly.

Two controls were run routinely. The first was a saline (DS) control and was well 9 for each SF test. For this control to be acceptable, there could be no agglutination or cell lysis during the course of the experiment. The second was a control for the surface antigen of the amoeba used in the agglutination test. This control consisted of a spot plate, like that described above, in which anti-amoeba rabbit serum was substituted for SF. This antiserum was obtained from a rabbit that had been injected repeatedly with purified plasma membranes from Acanthamoeba castellanii, strain I-12. It was of high titre and prepared in 1972. The method of raising anti-amoeba plasma membrane antibodies has been described by others (24).

An indirect test for involvement of complement, i.e., the lytic reaction, was performed by heating SF or serum at 57° C for 20 min before use in the spot test as described above.

Serum samples, to be used for comparison with SF in both agglutination and lysis studies, were obtained from the American Red Cross Blood Bank, Nashville, Tennessee. Each serum was tested as described for SF.

Thermal migration. Wyburn-Mason's setup (26, pp 120-121) for thermal migration was reproduced in the laboratory. The identical filter he used (Oxoid London, average pore size .8 micron) was obtained. His procedure was followed as closely as possible. Other setups were devised in the present study so that cells had to move through the filter if they got to the warm side of the membrane. These setups are described in the Results section.

Distribution of work. All microscopic examinations of fresh and stained materials as well as the routine examination bacterized SAA plates were performed by the principal investigator. The following duties were performed primarily by the research assistants: preparation of media; inoculation of media; smearing and staining of
slides; routine examination of cultures; maintenance of stock cultures; preparation and sending of letters of request for samples and terminal reports to physicians submitting samples; library work. Both principal investigator and research assistants cleaned and sterilized glassware and kept three growth rooms clean and functional.

RESULTS.

Solicitation and sources of SF. All U. S. members of The Rheumatoid Disease Foundation were asked to submit samples of SF during the late Summer and Fall of 1985. Letters to them contained Sample and Patient Information forms as well as mailing labels. Sample tubes were provided when requested. Early in 1986, some local physicians were asked to submit samples also. In all, 35 samples of SF were contributed by 15 physicians. Twelve physicians were members of The Rheumatoid Disease Foundation and three were local. The Rheumatoid Disease Foundation physicians contributed 25 of the samples, locals 10. Samples were received from 11 states. The states and number of samples were: Alabama (5); California (6); Florida (2); Indiana (3); Mississippi (2); New Jersey (1); New York (1); Ohio (1); Pennsylvania (1); South Carolina (3); Tennessee (10).

Types of arthritis, anatomical site of origin of SF, and duration of the arthritic condition. The types of arthritis, of patients from which SF was obtained, as identified by the submitting physician, are given in Table 1. The majority, 18 of 25, was listed as rheumatoid arthritis. The anatomical sites from which the samples were obtained are given in Table 2. In the majority of cases, 25 of 35, the sample was taken from the knee. The duration of the arthritic condition is given in Table 3. The majority of cases, 20 of 35, were of long duration, more than 1 year. Physician reports on drugs administered prior to sampling, were so incomplete that they are not reported here.

Microscopic identification of constituents in fresh preparations. Soil amoebae were not found by direct microscopic examination in any fresh preparation of SF. White blood cells were found in all of the samples, red blood cells in 26. In 3 of the 35 all white cells were judged to be dead. In the remaining samples, 10 to 100% of the white cells were alive when received. Cells capable of extending pseudopods varied from about 2% to 40% during observation at 29°C. White blood cells showed a remarkable ability to stay intact, at all temperatures and in or on all media used — including bacterized SAA. At the end of the month of culturing all, or most, of the cells in liquid media were dead as identified by phase but were still identifiable. In 2 cases, white cells were able to extend pseudopods after storage in the refrigerator for a month at 4°C. It was mainly the larger mononuclear cells that were able to attach to the floor of the counting chamber. Their characteristic large round nucleus, without identifiable nucleolus, and their ruffled pseudopods, permitted live identification as monocytes or macrophages. However, small nucleated cells, frequently with pseudopods, could not be identified with certainty as either small amoebae or small white blood cells. Differentiation required Giemsa staining of thin smears.

Red blood cells could always be identified with phase contrast at 400 X. Red blood cells were found in 26 of the 35 samples of SF. In 21 samples, some or most of the red cells had bizarre shapes due to extending barbs or knobs. These were not crenated forms as sometimes seen in normal blood. These shapes were retained after dilution in isotonic saline or after smearing on slides.

Cysts were not seen in SF. Living cells from SF that had been diluted or washed with PS or BSS, and observed at daily intervals, never formed cysts. Acanthamoeba strains encysted to some degree, usually within a day, in either of these salines.
A common feature of the non-cellular portion of the SF was the presence of fibres, easily seen with phase contrast. Twenty eight of the samples showed these fibres. They were not recorded in the first 7 samples received. They may have been missed in these early samples. Fibres were .2 to .5 microns thick and variable in length. Some were straight, some had sharp bends and a few looked like damaged coiled springs. Fibres occurred in bundles and sometimes had numerous macrophages embedded in them. The larger bundles could be seen easily by eye. The fibres resembled those commonly seen in sections of fibrous connective tissue. Fibres were extremely persistent. In one tube of inoculated NGM that got misplaced in the 290°C growth room, they were still easily identifiable after 7 months. The fibres showed weak positive birefringence with respect to their long axes.

Stained preparations. Amoebae were not identified in any of the stained preparations of SF. It was possible to classify the white cells in SF smears even in samples where most or all of the cells were dead at the time of smearing. The differential staining was usually off in cells that were dead when smeared. Cells identified were large monocytes, lymphocytes, and polymorphonuclear neutrophils. Immature leukocytes, eosinophils, basophils, or platelets were not identified in SF. The predominant types of white blood cells found in the samples of SF of this study are shown in Table 4. Clearly there is not a single white blood cell picture for the SF from these patients. The presence of abundant numbers of neutrophils and monocytes point to the inflamed condition of most of the joints from which SF was obtained.

Attempts to find chromatin-type inclusions in the barbed and knobby red blood cells, with the three stains used, was not successful. Nor did the fibres stain appreciably. With Giemsa's, fibres stained a light pink or purple and were very difficult to see in brightfield.

Findings from culture studies. Amoebae were not found in any of the cultures of SF. The &y organisms cultured were 2 fungi and 4 bacteria. These were isolated from the first few samples and probably resulted from the developing transfer and handling skills of the research assistants. No structures that resembled cysts were found in any of the cultures.

In three samples tested in tissue culture media, large monocytes attached, In one sample two small colonies of fibroblasts were found during the third week of culture. Some of the white blood cells were intact at the end of the third week.

The validity of the amoeba culture methods, used in this research, should be indicated. First, as controls on the quality of each medium used, the in-house soil amoebae were inoculated into the various media at regular intervals. New batches of media were tested with laboratory organisms before use with SF samples. Secondly, late in the grant period, the principal investigator was asked by the Department of Ophthalmology of Vanderbilt University Hospital to test samples from six patients with corneal keratitis suspected to be due to a virus or an amoeba. Methods of the present study were used. From two of the patients amoebae were isolated from corneal scrapings, soft contact lenses worn by the patient, and from saline used to clean the contact lenses. Amoebae from the patients were obtained in pure culture in NGM and submitted to CDC for identification. Morphologically the trophozoites and cysts were more like Acanthamoeba than Naegleria.

Testing the thermal migration method. A month and a half was spent testing Wyburn-Mason's thermal migration method. His setup was reproduced. It consisted of a funnel with a rubber tube at the bottom which could be clamped shut. A circular zinc mesh screen was wedged in about half way up the funnel bowl. On top of the screen was placed a round sheet of cellulose-acetate membrane filter several centimeters greater
in diameter than the zinc screen. The edges of the filter were folded so the center circle of the filter covered the zinc screen. To operate the device, the rubber tube was clamped shut, PS was placed in the bottom of the funnel to slightly above the level of the zinc mesh, and the device was suspended in a constant temperature water bath at 37° C to the level of the zinc support. Minced tissue (or SF) was placed in the filter and the bottom of a beaker of ice placed in contact with the biological material. Migration was allowed for 1 to 2 hours after which the PS was drained into a tube, cells centrifuged down, or allowed to settle, and the concentrated cells examined or cultured.

The setup in the present study was very similar. However, a zinc screen could not be found, and a stainless steel plate was substituted. Also, for the test amoebae studied, a 29° C bath was used. Both Naegleria and Acanthamoeba were tested but the I-3B2 strain of Acanthamoeba was used most. PS was used in most experiments but BSS worked at least as well.

Results of the first experiments with the Wyburn-Mason setup were mixed. In some, variable numbers of amoebae were found in PS. In others no amoebae were recovered. It was considered possible that amoebae found in the PS were not going through the pores but rather through cracks in the folds of the filter, or were being helped over the edge of the filter by capillarity or by agitation of the sample during ice changes. Oxoid London, manufacturers of the filters, indicate the filters crack or tear easily when folded, even when wet. The filters are strong and essentially immune to cracks when maintained in the flat condition and supported.

To determine if amoebae were actually going through the pores of the filter, membrane filtration funnels, manufactured by Millipore Corporation, were used. These devices are used to filter-sterilize liquid media. They are so constructed that materials can go only through the pores of the membrane filter. Two types were used, glass and stainless steel. Results were identical with both. Only the glass filter funnel setup will be described. Essentially it is a funnel cut in half. It consists of two parts. The first is a scinttered glass base and consists of the funnel stem and the lower part of the funnel bowl. To the top of this base, the manufacturer fused the edges of a plate of scinttered (porous) glass and then ground the plate and edges flat. The second part consists of the upper part of the funnel bowl, the bottom edge of which was ground flat so as to fit the edge of the scinttered-glass base. In use, the membrane filter was placed on the scinttered surface and the upper and lower portions clamped together tightly around the edge of the filter. This setup is used routinely to render tissue culture media sterile when used with .22 or .45 micron pore filters.

In the present work a rubber tube was attached to the tip of the base, the base filled with PS or BSS, the rubber tube clamped shut, and the membrane filter clamped between the base and funnel top. The whole assembly was lowered in a water bath until the membrane was at the water level of the water of the bath, a cell suspension added on top of the membrane, a flat-bottomed tube of ice placed in contact with the cell suspension, and migration permitted for 1 to 8 hours.

Using membranes with .8 micron pores (the same brand and pore size used by Wyburn-Mason) in the Millipore setup, amoeba migration through the membrane into the warm PS never occurred. Membrane filters with pore sizes of .45, 1.2, 8.0, and 14.0 microns (Millipore, cellulose nitrate) were tested. Amoebae migrated through the membranes, along the thermal gradient, but only through membranes with pore sizes of 8.0 and 14.0 microns. Migration was slow through the 8.0 but rapid through the 14.0 micron pores. A total of three SF samples were tested. Each contained many living cells. They were tested at 37° C using the .8 micron pore membrane filter, for 3 to 4 hours each. Cell migration was not observed with any of the samples. The failure of the
Wyburn-Mason method led to the concentration of cells, in the remainder of the study, by centrifugation or settling of dilute samples.

**Immunological studies.** Twenty six samples of SF and 27 samples of normal human serum were tested for their ability to agglutinate or lyse living Acanthamoeba cells, strain Ic-3B2. A summary of this data is presented in Table 5.

Agglutinating antibodies were detected in all samples tested. Quantitatively, the average titre of agglutinating antibodies determined in normal human serum, 56, was 4 times as great as that found in SF, 14.

Complete or 100 % lysis was found with all samples of normal human serum. The average titre for 100 % lysis was 5 and that for any lysis was 9. For SF, the picture was different. Only 2 of the 26 samples produced 100 % lysis and only 16 of the 26 produced some cell lysis. That is, 10 of the 26 samples produced no cell lysis, even at the smallest dilution of 1:2. Interestingly, the average titre in those 16 SF samples showing some lysis was not remarkably different, 6, from the average titre of all the human sera, 9.

All samples with lytic ability, both SF and human serum, lost the ability after heating at 56 °C for 20 min. Agglutination ability remained after heating. This strongly suggested that lysis was mediated by complement (C). Some components of C are heat labile at 56 °C. Lysis of all strains of Acanthamoeba and Naegleria carried in the laboratory, as well as the two clones of amoebae isolated from patients with amoeba corneal keratitis, were lysed at a 1:2 dilution of all samples of normal human serum tested.

**DISCUSSION.**

**Validity of the present findings.** A major result of the present study was that amoebae were not found in any of the 35 samples of rheumatoid disease SF examined. This is in direct contradiction to the reported findings of Wyburn-Mason (25, 26, 27) in which amoebae were identified and isolated in all samples. Validation of the current findings and comparison with those of Wyburn-Mason follow.

The procedures used in the present study were selected so that: any cell or structure of microscopic dimensions could be seen in fresh preparations of SF or of growth medium with phase contrast microscopy; any amoeba could be seen and differentiated from any host cell by brightfield microscopic examination of smears of SF, stained differentially with Giemsa's stain; any living amoeba of the genera Acanthamoeba or Naegleria, pathogenic or free-living, could be cultured and brought into pure or axenic culture in one or more of the media used; and any birefringent object of fresh preparations could be found and its sign of birefringence determined by polarization microscopy.

**Direct microscopic examination with phase contrast microscopy.** Wyburn-Mason's primary microscopic method of identifying amoebae seems to have been by observation of wet preparations of those cells that came through the membrane filter of his thermal migration setup. He did not say what type of microscope he used. Judging from the lack of structural definition in his published photomicrographs, he was using bright-field optics. Little of living structure can be seen with brightfield microscopy. Therefore, it would have been difficult or impossible for him to distinguish between amoebae and host cells. This is due to the fact that visualization of structure in brightfield depends primarily on absorption of light by colored structures. Since most cells and their components are uncolored, most structures in living cells are not seen in brightfield. On the other hand, visualization with phase contrast optics depends
primarily on small differences in refractive index between a structure and its surroundings. Thus, essentially any structure of microscopic dimensions, in or out of cells, can be seen. Mature cysts could be identified in brightfield.

In the present study, it was difficult to identify any of the white cells with certainty or to distinguish them from amoebae with bright field. With phase contrast, at 400 X, and using a counting chamber, spread amoebae, spread macrophages, bacteria, fibres, cysts, and fungi could be identified readily. Rounded lymphocytes and rounded polymorphonuclear leukocytes could not be distinguished from small rounded amoebae in the counting chamber. These cells could be identified in thin slide-coverslip preparations where the cells were flattened by pressure. Nuclear structures and cell organelles could be resolved in these thin preparations. Few slide-coverslip preparations were made because of cell breakage. In practice, the counting chamber was used to make total cell counts in which red and white blood cells were distinguished. It was also in the counting chamber that searches were made for cysts, fibres, crystals, bacteria, yeast, etc. No amoebae, cysts, or fragments of either, were found in the examination of any of the 35 SFs.

Examination of thin stained smears of SF with brightfield microscopy. The only staining Wyburn-Mason seemed to have done of cells isolated by his thermal migration method was with the PAS (periodic acid Schiff) stain. This procedure stains insoluble polysaccharides mainly, gives diffuse cytoplasmic staining, if any, and provides no nuclear staining. In the present study, final differentiation between white cell types and amoebae was done by examining, in brightfield at 1,000 X, smears of SF processed by a carefully controlled Giemsa staining procedure. Nuclear and cytoplasmic constituents of white cells and amoebae stained in different colors and shades of color so the identification of cell types was facilitated. With this method all white cells and amoebae could be identified provided the smeared cells were flat. No amoebae, trophozoites or cysts, were found in any of the SF smears.

Attempts to culture amoebae from SF. Frequently, pathogenic microorganisms cannot be identified during microscopic examination but can be grown in suitable media. In the present study, media used to cultivate both pathogenic and free-living amoebae, FBM and NGM, were used. Also, a bacterized plate method, identical to the one Wyburn-Mason claimed to have used to isolate amoebae from all rheumatoid disease patients, was used. The utility of these three media was proven by successfully isolating Acanthamoeba from 2 of 6 cases of corneal keratitis.

Two other media were used. FTM was included since it is commonly used to test for sterility. It also supports growth of Acanthamoeba but not Naegleria. The other medium, SDA, was used to screen for fungi. Amoebae are not known to grow on SDA. No amoeba growth was found in or on any of the media, for any sample, at any of the three temperatures used.

Search for cysts. Wyburn-Mason described cysts (26) of unusual appearance as well as their formation from amoebae immersed in amoeba saline. His descriptions stimulated the search for cysts in the SF samples of the present study. If cysts had been present, they would have been identified with all of the microscopic methods used - most easily with polarization microscopy. Cyst walls of amoebae contain cellulose including those of Chaos chaos, the giant amoeba. If cysts had been present, they, or the cellulose in their walls, would have persisted and should have been observed. Digestion of cellulose by mammalian cells has not been demonstrated. Acanthamoeba form cysts in tissue, while Naegleria form few if any cysts in tissue. Cysts were not found by microscopic or growth methods. Nor did cysts form in saline solutions as described by Wyburn-Mason.

If not cysts, what were the structures Wyburn-Mason saw and called cysts? His photo-
micrographs (26, pp 122-123) showed empty structures that did not have the thickened walls characteristic of Acanthamoeba and Naegleria cysts. Structures found in stained slides, in the present study, did resemble the structures which Wyburn-Mason called cysts. They are known as basket cells by hematologists (6). Basket cells are nuclei of degenerating lymphocytes or monocytes. In them the chromatin has clumped forming fibrous or rope-like strands. This gives a structure which has a reticular or "fenestrated" appearance. With basket cells, it is usual that the cytoplasm has broken away from the nucleus. Further, basket cells stain like nuclei. If Wyburn-Mason's cysts were in fact host cell nuclei, how did they get into the PS? The present study has shown that cells and debris went through cracks or over the sides of membrane filters in Wyburn-Mason's thermal migration apparatus. It seems likely that when Wyburn-Mason minced tissue, host cells and host cell nuclei were released and entered the saline. Once in the saline, the divalent cations would cause the nuclear chromatin to clump and the nuclei to stick together. Perhaps this chromatin clumping was mistaken for encystment. Chromatin clumping is well known to students of the cell that break open cells and fractionate the organelles in non-ionic solutions such as sucrose. As long as nuclei remain in sucrose they look like living nuclei and have the same consistency. However, on adding divalent cations the structure of the chromatin becomes fibrous and nuclei stick to each other - sometimes in large clumps. It seems most likely that the structures identified by Wyburn-Mason were damaged host cell nuclei, not cysts.

Conclusions about the microscopic and growth findings. It now seems reasonable to conclude that the methods used by Wyburn-Mason did not permit him to differentiate microscopically between host cells and amoebae. Further, it is unlikely that he grew amoebae on bacterized plates. It is more likely that he observed blood cells that persisted but did not grow or divide. Also, it now seems probable that the structures he called cysts were damaged and clumped host cell nuclei. It is firmly believed that the methods used in the present study permit the conclusion that amoebae were not present in any of the SFs tested.

On the other hand, it would be incorrect to conclude from the present data that amoebae were never in the joint or that amoebae are not the cause of rheumatoid disease. It is possible that amoebae do invade joints and set off an inflammatory response that results in the destruction of the amoebae and much of the joint tissue of the host. Had amoebae or cysts been in the joints at earlier times, one might expect to find pieces of trophozoites or of cysts. It is possible, of course, that dead trophozoites were phagocytosed by macrophages or neutrophils. An amoeba that did not form a cyst may well have had all of its remains removed. If so, is there any way to determine if amoebae once inhabited the rheumatic joint? Possible methods of answering this question are considered below.

Immunological studies. After the battle, between a host immune system and an invading microorganism, all visible remains of the invader may have been removed by host scavenger cells. Evidence of the conflict may still remain in the form of serum antibodies specific against the microorganism. Such a consideration led to the preliminary immunological work reported in the present study. In it, the amoeba-agglutination-antibody titres and the lytic titres were determined for each of 26 rheumatoid disease SFs and 27 normal human sera.

Normal human sera. The present study showed that antibodies to cell surface antigens, of two genera of soil amoebae, were present in appreciable concentrations in all normal human sera. This is consistent with observations of others (4, 5). These data suggest that all members of the human population have been exposed to soil amoebae in intimate ways and repetitively. It was also found, in the present study,
that all human sera contained heat labile components capable of in vitro killing or lysis of all soil amoebae tested. This suggests all humans with intact immune systems should be immune to soil amoebae.

There are two known immune mechanisms by which amoebae can be killed. The first is humoral and involves C-mediated lysis. There are two C pathways. In the classical C pathway, specific antibodies to the invader are required for lysis. In the alternate C pathway, antibodies are not required. Any cell without sialic acid residues on its surface may trigger the alternate C pathway (7). Detailed studies by Australian workers (8) indicate that the killing of pathogenic Naegleria and Acanthamoeba is by the alternate C pathway. It is not known whether the classical or alternate C pathway, or both, was at work in the present study. The second method of cell killing is cell mediated. The Australian group has shown that pathogenic amoebae can be killed by a cellular lytic mechanism (5, 9, 10, 23). In this case, both amoeba antibodies and neutrophils, activated by a macrophage lymphokine, are required. Because of the antibody requirement, both the cell mediated mechanism and the classical C pathway are very specific lytic reactions.

Synovial fluid. Both the amoeba-agglutination-antibody titre and the lytic titre of the SFs were found to be reduced as compared to the same activities in normal human sera. The low antibody titre was surprising. One might expect a tissue attack by an amoeba would result in higher serum antibody titres. Higher serum antibody titres may actually be the case in the present study since sera of the patients were not available for testing. Only the patient SFs were tested. The low SF titres found could be due to two mechanisms. The first would be the depletion of amoeba antibodies in the SF of the afflicted joint but not in the serum. The second would be the suppression of the immune system. In this case the antibody level would be low in both SF and serum.

Although there are no other papers on titres of amoeba antibodies in SFs of rheumatoid disease patients, there are many dealing with variations in concentrations of plasma proteins, including C and immunoglobulins, during development of inflammation and effusion into rheumatoid joints (1, 2, 19, 20). In general, the concentration of plasma proteins in SF is about one-fourth of that found in blood but increases and approaches that of blood during inflammation. C and immunoglobulins increase in the joint, in parallel with total blood proteins, unless they are depleted by some immune process. Activation of the classical C pathway or neutrophil activation would produce such an antibody depletion process. In the case of infection by known pathogenic amoebae of the genus Acanthamoeba, there is good evidence that these amoebae invade individuals with deficient or suppressed immune systems (14, 15, 22). There seems to be no evidence for widespread immune deficiency in rheumatoid disease cases. Thus, it may be that the immune system is not deficient but only depleted in a localized region. By contrast, Naegleria are able to infect individuals with vigorous and intact immune systems (14, 15).

The reduced level, or complete absence, of heat labile lytic power in some SFs was a new finding. However, there are papers in the literature that are consistent with this result. In particular, Britton and Schur (1) quantitated the amounts of various components of blood plasma found in SF. The materials studied included elements of the C system, members of the immunoglobulin fraction, serum albumin, and rheumatoid factor (RF). These authors also looked for the same materials in these phagosomes of SF leukocytes obtained from patients with RF+ and RF- rheumatoid arthritis (RA) and patients with degenerative joint disease (DJD). The C and immunoglobulin concentrations in the SF of RF+ patients were very low and aggregates of immunoglobulins (especially IgG and IgM) and C were found in the phagosomes of their leukocytes. By contrast,
the RF- and DJD had elevated C and immunoglobulin concentrations in their SFs comparable with those in serum. Only small numbers of the RF-, SF leukocytes contained aggregated C and immunoglobulins. These workers did not look for specific antibodies in the immunoglobulin fraction. They proposed that their findings were consistent with activation of the C system and with phagocytosis of immune complexes by the SF leukocytes.

Research prospects. Results of the present work, considered in the light of the work of Britton and Schur (1) and the Australian workers (5, 8, 9, 10, 23), open the possibility that immune aggregates, phagocytosed by leukocytes in the SF of RA patients, might be enriched in antibodies against an invading organism. If phagosomes actually contain enriched concentrations of amoeba antibodies, it would suggest amoebae had invaded the joint previously. Too, one might expect to be able to pellet activated neutrophils from SF which could attack soil amoebae exposed to specific antibodies.

Research projects which might provide circumstantial evidence for the previous presence of soil amoebae in RA joints are the following:

1. Determine the concentration of amoeba antibodies in both the SF and serum of RA patients. The enrichment of the antibody might be determined by comparing the antibody concentration with the concentration of a non-immune constitutnt such as human serum albumin.

2. Determine if amoeba antibody complexes with antigen and C fragments are present in the phagosomes of leukocytes of a series of both RF+ and RF- RA patients. If present, determine if the antibodies are enriched in the phagosome aggregates as compared to other antibodies or other proteins found in the same SF.

3. Determine if neutrophils, present in both SF and peripheral blood, are already activated to attack amoebae and if the attack is mediated by amoeba antibodies.

4. Determine the complement pathway/s present in SF and in serum.

General conclusions from the present study. Amoebae, trophozoites or cysts, were not found in SFs of RA patients. The cells which Wyburn-Mason isolated and believed to be amoebae, were in all likelihood host cells. Most, or all, were white blood cells. The thermal migration method, as practiced by Wyburn-Mason, did not isolate the putative Amoeba chromatosa from host cells but rather permitted host cells and host cell debris to go through cracks in the filter or over the edge of the filter. The structures called cysts by Wyburn-Mason, are believed to be host cell nuclei damaged by saline. In all likelihood, they are the basket cells of the hematologists. Antibodies that agglutinate soil amoebae and an amoeba-lytic principle were found in high concentrations in all normal human sera. Antibodies were reduced while the lytic principle was reduced or absent in the SFs of rheumatoid disease patients. It was proposed that a last place to search for evidence that supports the hypothesis that amoebae are the causative agent of rheumatoid disease is in the SF leukocyte. The evidence, if found, would be enriched concentrations of aggregates of amoeba antibodies in SF leukocyte phagosomes and antibody-directed-anti-amoeba neutrophils.
Table 1. Type of arthritis.*

<table>
<thead>
<tr>
<th>Type</th>
<th>Number of patient samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid arthritis</td>
<td>18</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>2</td>
</tr>
<tr>
<td>Psoratic arthritis</td>
<td>2</td>
</tr>
<tr>
<td>Traumatic arthritis</td>
<td>2</td>
</tr>
<tr>
<td>Gouty arthritis</td>
<td>1</td>
</tr>
<tr>
<td>Gout</td>
<td>1</td>
</tr>
<tr>
<td>Synovitis</td>
<td>1</td>
</tr>
<tr>
<td>Unspecified</td>
<td>8</td>
</tr>
</tbody>
</table>

* As indicated by the physician submitting the sample.

Table 2. Anatomical site of origin of synovial fluid samples.

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of patient samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knee</td>
<td>25</td>
</tr>
<tr>
<td>Shoulder</td>
<td>2</td>
</tr>
<tr>
<td>Wrist</td>
<td>1</td>
</tr>
<tr>
<td>Unspecified</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 3. Duration of the arthritic condition.

<table>
<thead>
<tr>
<th>Duration</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than six weeks</td>
<td>5</td>
</tr>
<tr>
<td>Six weeks to one year</td>
<td>2</td>
</tr>
<tr>
<td>Longer than one year</td>
<td>20</td>
</tr>
<tr>
<td>Unspecified</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 4. Predominant type of white blood cell in the synovial fluid.

<table>
<thead>
<tr>
<th>White blood cell type</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>7</td>
</tr>
<tr>
<td>Monocytes</td>
<td>12</td>
</tr>
<tr>
<td>Polymorphonuclear neutrophils</td>
<td>6</td>
</tr>
<tr>
<td>Approximately equal mixtures of:</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes and monocytes</td>
<td>1</td>
</tr>
<tr>
<td>Lymphocytes, monocytes, neutrophils</td>
<td>2</td>
</tr>
<tr>
<td>Lymphocytes and neutrophils</td>
<td>1</td>
</tr>
<tr>
<td>Predominant type not determined</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 5. Summary of data on agglutination and lysis of Acanthamoeba.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Synovial fluid from rheumatoid disease patients</th>
<th>Normal human serum.(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agglutination</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Number of samples showing agglutination</td>
<td>26 of 26</td>
<td>27 of 27</td>
</tr>
<tr>
<td>2. Average agglutination titre(^d)</td>
<td>14</td>
<td>56</td>
</tr>
<tr>
<td>3. Smallest titre at which agglutination was observed in any sample</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>4. Largest titre at which agglutination was detected in any sample</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td><strong>Lysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Number of samples showing 100 % lysis</td>
<td>2 of 26</td>
<td>27 of 27</td>
</tr>
<tr>
<td>6. Number of samples showing any lysis</td>
<td>16 of 26</td>
<td>27 of 27</td>
</tr>
<tr>
<td>7. Average titre in samples indicated</td>
<td>6 (of the 16 samples showing lysis)</td>
<td>9 (of 27 samples)</td>
</tr>
<tr>
<td></td>
<td>2 (all 26 samples)</td>
<td></td>
</tr>
<tr>
<td>8. Average titre for 100 % lysis in the samples indicated</td>
<td>less than 1 (26 samples)</td>
<td>5 (27 samples)</td>
</tr>
<tr>
<td></td>
<td>4 (the 2 samples showing 100 % lysis)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Strain I\(^2\)-3B2.
\(^b\) A total of 26 samples.
\(^c\) A total of 27 samples.
\(^d\) The titre of agglutination is the reciprocal of the maximum dilution at which agglutination can be detected (7). If the maximum dilution was found to be 1:64 then the titre would be 64. Also, the titre is directly proportional to the concentration of the antibody in a given serum. Therefore, the relative concentration of antibodies in different sera can be assessed by comparing titres.
REFERENCES.


